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**Title:**

Plasma glucagon-like peptide-1 responses to ingestion of protein with increasing doses of milk minerals rich in calcium

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**Author contributions:**

JW, JG, LHB and URM designed the research, JW conducted the research, HS and AH prepared the test meals, JW and HS analysed the data, JW performed the statistical analysis, JW primarily wrote the paper and all authors read and approved the final version of the manuscript.

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**Running Title:**

Protein, calcium and GLP-1 release

**Keywords:**

Calcium; Protein; GLP-1; Appetite; Energy intake

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**CONFLICTS OF INTEREST**

JTG is a named investigator on research grants funded by BBSRC, MRC, British Heart Foundation, Lucozade Ribena Suntory, Arla Foods Ingredients, Kenniscentrum Suiker and Voeding; and has received paid consultancy for PepsiCo, Lucozade Ribena Suntory and SVGC.

JAB is a named investigator on research grants funded by BBSRC, MRC, British Heart Foundation, Rare Disease Foundation, EU Hydration Institute, GlaxoSmithKline, Nestlé, Lucozade Ribena Suntory, ARLA foods and Kennis Centrum Suiker; has completed paid consultancy for PepsiCo, Kellogg's and SVGC; receives an annual stipend as Editor-in Chief of International Journal of Sport Nutrition & Exercise Metabolism; and receives an annual honorarium as a member of the academic advisory board for the International Olympic Committee Diploma in Sports Nutrition.

## ABSTRACT

A high dose of whey protein hydrolysate fed with milk minerals rich in calcium (Capolac®) results in enhanced glucagon-like peptide-1 (GLP-1) concentrations in lean individuals, however the effect of different calcium doses ingested alongside protein is unknown. The present study assessed the dose response of calcium fed alongside 25 g whey protein hydrolysate on GLP-1 concentrations in individuals with overweight/obesity. Eighteen adults (mean  $\pm$  SD: 8M/10F, 34  $\pm$  18 years, 28.2  $\pm$  2.9 kg·m<sup>-2</sup>) completed 4 trials in a randomised, double-blind, crossover design. Participants consumed test solutions consisting of 25 g whey protein hydrolysate (CON), supplemented with 3179 mg (LOW), 6363 mg (MED), or 9547 mg (HIGH) Capolac® on different occasions, separated by at least 48 hours. The calcium content of test solutions equated to 65, 892, 1719 and 2547 mg, respectively. Arterialised-venous blood was sampled over 180 min to determine plasma concentrations of GLP-1<sub>TOTAL</sub>, GLP-1<sub>7-36amide</sub>, insulin, glucose, non-esterified fatty acids (NEFA), and serum concentrations of calcium and albumin. *Ad libitum* energy intake was measured at 180 min. Time-averaged incremental area under the curve (iAUC) for GLP-1<sub>TOTAL</sub> (pmol·L<sup>-1</sup>·min<sup>-1</sup>) did not differ between CON (23  $\pm$  4), LOW (25  $\pm$  6), MED (24  $\pm$  5), and HIGH (24  $\pm$  6). Energy intake (kcal) did not differ between CON (940  $\pm$  387), LOW (884  $\pm$  345), MED (920  $\pm$  334), and HIGH (973  $\pm$  390). Co-ingestion of whey protein hydrolysate with Capolac® does not potentiate GLP-1 release in comparison to whey protein hydrolysate alone. The study was registered at clinical trials (NCT03819972).

## INTRODUCTION

Glucagon-like peptide-1 (GLP-1) is a peptide hormone produced by intestinal L-cells<sup>(1)</sup>. Its release stimulates glucose-dependent insulin secretion<sup>(2)</sup>, delays gastric emptying<sup>(3)</sup>, and reduces food intake<sup>(4)</sup>. These actions, among others, act to preserve metabolic health such that GLP-1 agonism has been targeted for obesity and type 2 diabetes (T2D) management<sup>(5)</sup>. Strategies including bariatric surgery, administration of GLP-1 agonists, and dipeptidyl peptidase-IV inhibitors (DPP4 rapidly inactivates GLP-1 upon binding) have been effective at increasing/preserving GLP-1 action and promoting weight loss and greater glycaemic control<sup>(5; 6; 7; 8)</sup>. However, it is also important to understand the regulation of endogenous GLP-1 through nutrition, whereby enhancement of gut hormones through specific targeted nutrition can be implemented for wide-scale use.

Nutrition potently regulates enteroendocrine cell action and subsequent peptide hormone release through nutrient sensing and absorption<sup>(9)</sup>. Recent evidence has implicated the calcium sensing receptor (CaSR), which is expressed on intestinal L-cells, with facilitating gut hormone release across *in vitro*<sup>(10; 11)</sup>, *ex vivo*<sup>(12; 13)</sup> and *in vivo* rodent models<sup>(14)</sup>. CaSR is responsive to extracellular calcium and amino acids/peptides at physiological fasting and postprandial concentrations<sup>(15; 16)</sup>.

The administration of calcium and hydrolysed protein to stimulate GLP-1 secretion was first investigated using an *ex vivo* rodent model<sup>(12)</sup>. The small intestine was perfused with a KHB buffer in the presence or absence of 10 mM phenylalanine. Calcium was then introduced with increasing dose from 0.1 to 10 mM. GLP-1 concentrations were augmented with increasing calcium dose only in the presence of

phenylalanine <sup>(12)</sup>. More recent work in lean humans is consistent with these observations, whereby plasma GLP-1<sub>TOTAL</sub> iAUC was 25% greater over 2 h following acute ingestion of 50 g whey protein with Capolac® compared to the ingestion of 50 g whey protein alone <sup>(17)</sup>. Furthermore, peak GLP-1 concentrations measured following the ingestion of whey protein and Capolac® were some of the highest recorded in humans following nutrient ingestion ( $91 \pm 20$  pmol·L<sup>-1</sup>).

The previous study selected a large dose of protein as part of a proof of principle design. However, it remains unclear whether this effect persists in an overweight/obese population and when feeding a practically relevant dose of protein more reflective of that ingested during a standard meal i.e 25 g. For instance, total protein content of breakfast, lunch, and dinner for adults is approximately 15 g, 23 g, and 35 g, respectively <sup>(18)</sup>. Moreover, the dose of calcium required alongside protein to effectively stimulate the relevant gut hormone response is yet to be established. Accordingly, the aim of this study was to assess the dose-response of calcium fed alongside a practically relevant dose of protein on GLP-1 release in individuals who are overweight/obese. It was hypothesised that GLP-1 concentrations would rise with increasing dose of calcium.

## **METHODS**

### *Experimental design*

Eighteen overweight men and women (Table 1) were recruited to participate in a double-blinded randomised crossover study with 4 trial arms. Inclusion criteria included age 18-65 y, BMI 25.0-34.9 kg·m<sup>-2</sup>, no history of metabolic disease, and free from allergies or altercations to calcium or milk proteins. Following written consent,

participants were randomly assigned to a counterbalanced trial sequence by a researcher who was not involved in data collection. The study protocol was approved by the University of Bath Research Ethics Approval Committee for Health (REACH REF: EP 17/18 250). All procedures were carried out in accordance with the declaration of Helsinki. The study was registered at <https://clinicaltrials.gov/> (NCT03819972).

Table 1 Participant characteristics

	Total	Males	Females
Sample size (n)	18	8	10
Age (years)	34 ± 18	28 ± 14	38 ± 20
Body mass (kg)	81.2 ± 12.5	89.0 ± 12.3	74.9 ± 8.8
Body mass index (BMI; kg·m <sup>-2</sup> )	28.2 ± 2.9	29.1 ± 3.4	27.4 ± 2.4
Fasting glucose (mmol·L <sup>-1</sup> )	5.5 ± 0.5	5.5 ± 0.5	5.5 ± 0.7
Fasting insulin (pmol·L <sup>-1</sup> )	41 ± 20	43 ± 20	40 ± 21

Values are mean ± SD

#### *Pre-trial standardisation*

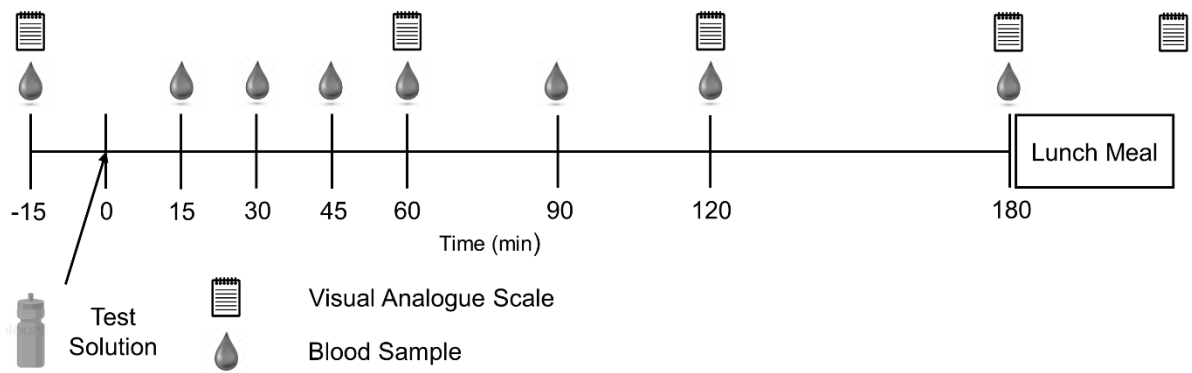
Participants recorded their habitual diet for 24 h prior to trial 1, then replicated this diet for 24 h prior to each subsequent trial. They also refrained from caffeine, alcohol and any vigorous physical activity for 24 h prior to each trial day. Participants were provided with a standardised meal (Tesco spinach and ricotta cannelloni 440 g; 463 kcal, 56.3 g carbohydrate, 10.1 g sugars, 25.0 g fat, 23.8 g protein) which was consumed no later than 10:00 pm the evening before a trial day. Women were tested at different phases of their menstrual cycle as this was considered most representative of real life. The washout period between trials was between 2-7 days.

## *Trial days*

Participants arrived at the laboratory between 08:00-10:00 h following a 10-14 h overnight fast (standardised within participants). Water consumption was permitted *ad libitum*, with volume recorded on the first trial, and replicated on the subsequent trials. Height was measured using a stadiometer (Seca Ltd., Birmingham, UK), with participants barefoot in the Frankfurt plane. Body mass was measured using digital scales (Tanita, Amsterdam, The Netherlands) with participants barefoot and wearing light clothing.

Arterialised-venous blood samples were obtained by retrograde cannulation of a pre-heated dorsal hand vein or, if unsuccessful, an antecubital arm vein (standardised within participants). After a baseline blood sample and visual appetite scale, test solutions were consumed within a 5-min window (trial commenced upon the first mouthful of the test solution). The time taken to ingest the test solution on trial 1 was recorded and replicated for each subsequent trial. Following ingestion, palatability and blinding validation scales were completed. Blood samples were taken at 15, 30, 45, 60, 90, 120, and 180 min post-ingestion. Appetite visual analogue scales (VAS) were obtained at baseline and every hour throughout the postprandial period. At the end of this period participants ingested an *ad libitum* lunch (Tesco Hearty Food Co cheese and tomato pasta 400 g; nutritional info per 100 g, 118 kcal, 19.9g carbohydrate, 15.9 g sugars, 6.8 g fat, 14.3 g protein) until they were comfortably full and did not want to consume any more. This was followed by another VAS and palatability scale. Upon the completion of the final trial participants completed a restrained eating questionnaire. The protocol is outlined in Figure 1.





**Figure 1.** Schematic of trial days

1/0

### Test solutions

There were 4 test solutions consumed in a randomised, counterbalanced order over 4 trials: 1) whey protein hydrolysate [29.8 g dry powder providing 25 g protein and 15 mg calcium; CON; Lacprodan® DI-3091; (Arla Foods Ingredients, Viby J, Denmark)]; 2) Whey protein hydrolysate plus milk minerals rich in calcium, Capolac® (Arla Foods Ingredients, Viby J, Denmark) [3179 mg to provide 842 mg calcium; LOW;]; 3) Whey protein hydrolysate plus Capolac® (6363 mg to provide 1669 mg calcium; MED;); 4) Whey protein hydrolysate plus Capolac® (9547 mg to provide 2497 mg calcium; HIGH;). Calcium concentrations for each condition increased linearly up to the maximal tolerable dose in HIGH. The calcium content in the local tap water was 99.58 mg·L<sup>-1</sup> <sup>(19)</sup> and day to day variation is <15 mg which is unlikely to alter any responses measured <sup>(17)</sup>. A tap water contribution of 50 mg calcium per 500 ml drink is included in the composition given in Table 2. Test solutions were prepared by two researchers not involved with data collection by dissolving both the protein and Capolac® together in 500 ml water. The composition of test meals is given in table 2.

189 Table 2 Nutritional composition of the test meal for each condition

Ingredient	Condition			
	CON <sup>a</sup>	LOW <sup>b</sup>	MED <sup>c</sup>	HIGH <sup>d</sup>
Energy (kJ)	438	444	449	455
Energy (kcal)	105	106	107	109
Water (ml)	500	500	500	500
Sucralose (mg)	80	80	80	80
Calcium (mg) <sup>e</sup>	65	892	1719	2547
Phosphorus (mg)	60	410	760	1110
Magnesium (mg)	15	37	60	82
Protein (g)	25	25	25	25
Carbohydrate (g)	0.6	0.9	1.1	1.4
Fat (g)	< 0.1	< 0.1	< 0.1	< 0.1

190 <sup>a</sup>CON, whey protein hydrolysate; <sup>b</sup>LOW, whey protein hydrolysate with a low dose of  
 191 Capolac®; <sup>c</sup>MED, whey protein hydrolysate with a medium dose of Capolac®; <sup>d</sup>HIGH,  
 192 whey protein hydrolysate with a high dose of Capolac®; <sup>e</sup>reported calcium value  
 193 includes the estimated tap water content.

194

# 195 *Blood sampling and analysis*

196 A 10-mL blood sample was collected for sampling of arterialised venous blood at each  
 197 timepoint. This was dispensed into 1) 5-mL EDTA collection tubes (Sarstedt,  
 198 Nümbrecht, Germany); 2) 3-mL untreated blood collection tubes (Sarstedt,  
 199 Nümbrecht, Germany); and 3) 2-mL p800 tubes (BD, New Jersey, USA) containing a  
 200 cocktail of enzyme inhibitors. Plasma samples (EDTA and p800 tubes) underwent  
 201 immediate centrifugation, whereas untreated blood collection tubes were allowed to  
 202 clot at room temperature for 15 minutes before being centrifuged to extract blood  
 203 serum. All samples were centrifuged at 4000 g for 10 minutes at 4°C and plasma and

serum collected and frozen immediately on dry ice. Samples were stored at -80°C until further analysis.

Plasma GLP-1<sub>TOTAL</sub>, GLP-1<sub>7-36amide</sub>, and insulin were measured using commercially available enzyme-linked immunosorbent assays (ELISA, GLP-1, Merck Millipore, Watford, UK; Insulin, Mercodia, Uppsala, Sweden). The antibodies in the GLP-1<sub>TOTAL</sub> assay are specific to both GLP-1<sub>7-36amide</sub> and GLP-1<sub>9-36amide</sub>, and therefore the GLP-1<sub>TOTAL</sub> assay is indicative of total secretion whereas the GLP-1<sub>7-36amide</sub> assay is indicative of hormone action <sup>(20)</sup>. All samples for comparison between treatments within each participant were included on the same plate and the respective intra-plate CVs for GLP-1<sub>TOTAL</sub>, GLP-1<sub>7-36amide</sub> and Insulin were 6.1%, 6.1%, and 7.4%, respectively. Plasma glucose and NEFA and serum calcium and albumin were analysed in singular using a spectrophotometric analyser (Randox, Daytona, Randox Laboratories Ltd., Crumlin, UK).

### *Subjective appetite ratings*

Subjective ratings of appetite were assessed using a previously validated 100-mm visual analogue scale (VAS) <sup>(21)</sup>. The four questions from this scale: 'How hungry do you feel?', 'How full do you feel?', 'How satisfied do you feel?', and 'How much do you think you can eat?' can be used and then converted into a composite appetite score. This score combines hunger, fullness, satisfaction, and prospective consumption using the following equation <sup>(22)</sup>.

$$\text{Appetite score} = (\text{hunger} + \text{prospective food consumption} + (100 - \text{fullness}) + (100 - \text{satisfaction}))/4$$

229

## 230 *Statistical analysis*

231 The primary outcome was the iAUC for plasma GLP-1<sub>TOTAL</sub>. Plasma GLP-1<sub>7-36amide</sub>,  
232 insulin, glucose and NEFA, and serum calcium and albumin were secondary  
233 outcomes, alongside VAS and *ad libitum* energy intake. The sample size calculation  
234 was based on our previous data<sup>(17)</sup> in which the mean difference for postprandial  
235 plasma GLP-1<sub>TOTAL</sub> between the co-ingestion of whey protein and Capolac<sup>®</sup> versus  
236 whey protein alone, was  $9.1 \pm 6.9$  pmol·L<sup>-1</sup>·120 min<sup>-1</sup> (mean  $\pm$  SD). Using this effect  
237 size ( $d = 1.32$ ), approximately 18 participants (16 participants for blood data) provides  
238 an 90% probability (85% for blood data) of detecting such an effect with an  $\alpha$ -level of  
239 0.05 using a one-way, repeated-measures ANOVA with two tails. Data are presented  
240 as means  $\pm$  SD in text, and means  $\pm$  95% normalised confidence intervals (nCI) in  
241 figures, corrected for between-participant variation, such that the magnitude of the  
242 nCI's directly infers the contrast between paired means at each time-point rather than  
243 variance of individual values around the mean <sup>(23)</sup>. The iAUC or total AUC (tAUC) was  
244 calculated for all variables (other than *ad libitum* energy intake) using the Time Series  
245 Response Analyser <sup>(24)</sup>. One-way ANOVAs were employed to assess differences  
246 between non-time dependent variables (i.e. iAUC, *ad libitum* energy intake), whereas  
247 differences between time-dependent variables were analysed using a 2-way repeated-  
248 measures ANOVA (condition-time). ANOVAs are considered robust to violations to  
249 normality assumptions <sup>(25)</sup>. Where data points were missing, mixed effects models  
250 were employed. Where an interaction effect was determined, *post-hoc* comparisons  
251 were adjusted for multiple comparisons using the stepwise Holm-Bonferroni  
252 correction. Simple linear regression was used to determine whether BMI and HOMA-  
253 IR could predict GLP-1 concentrations. A two-tailed  $p$  value of  $\leq 0.05$  was deemed

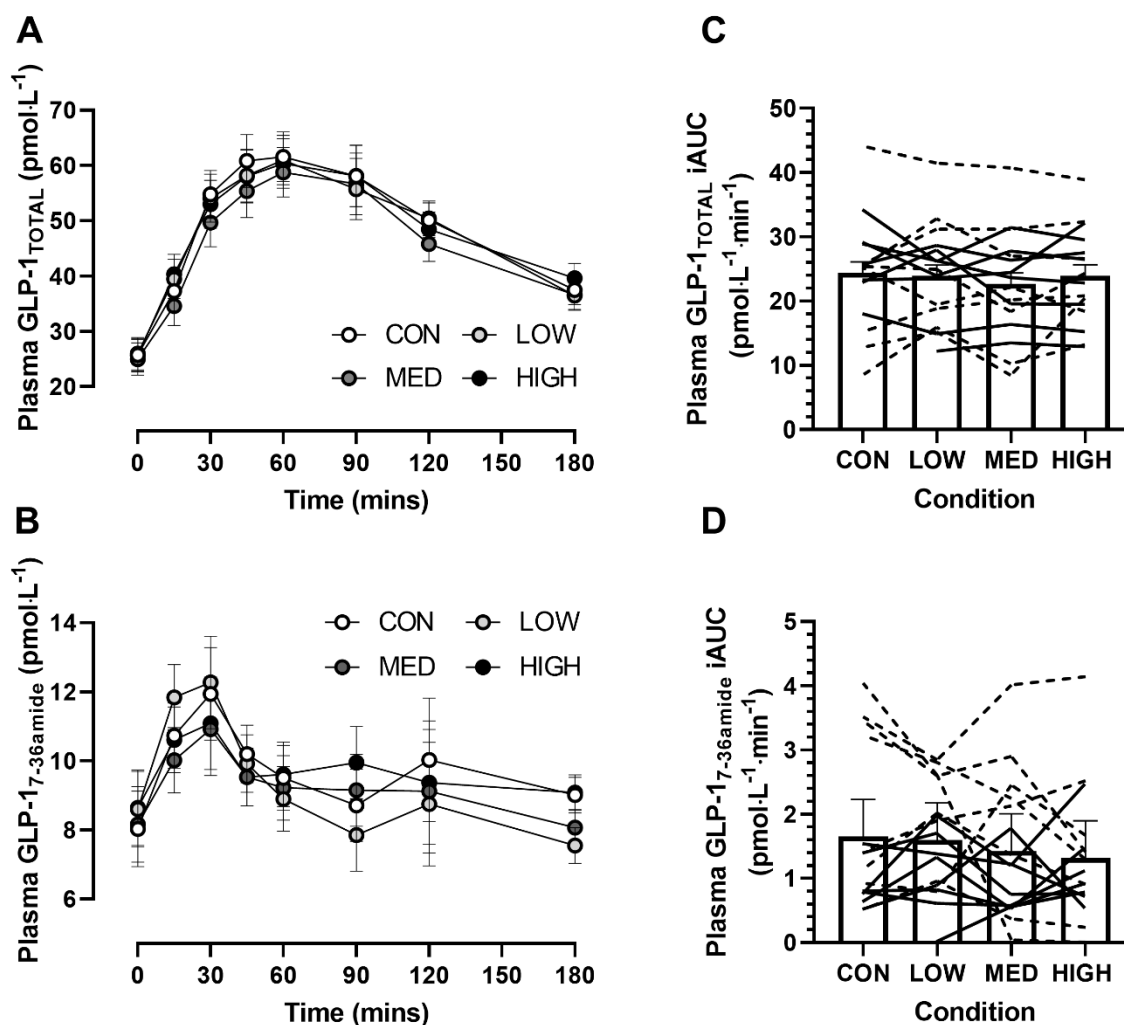
statistically significant. Results were analysed using Microsoft Excel version 16.0 and GraphPad Prism version 8.4.3.

## RESULTS

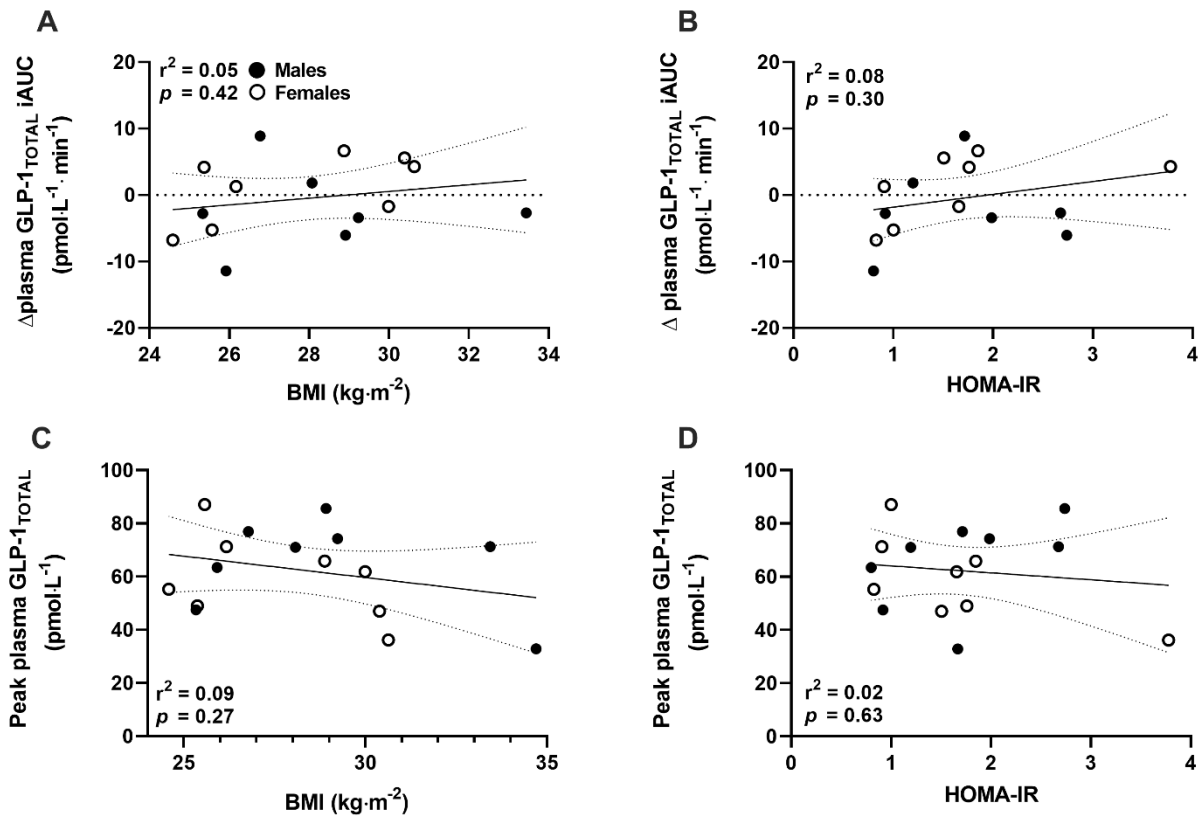
### *Plasma GLP-1*

For postprandial change in plasma GLP-1<sub>TOTAL</sub> and GLP-1<sub>7-36amide</sub> concentrations there was no main effect of trial ( $p = 0.24$  and  $p = 0.34$ , respectively) or trial x time interaction (Fig 2A-B;  $p = 0.22$  and  $p = 0.43$ , respectively). There was also no significant differences in plasma GLP-1<sub>TOTAL</sub> time-averaged iAUC (Fig 2C;  $p = 0.57$ , CON ( $24 \pm 9 \text{ pmol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$ ); LOW ( $24 \pm 8 \text{ pmol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$ ); MED ( $23 \pm 8 \text{ pmol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$ ); HIGH ( $24 \pm 7 \text{ pmol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$ )) and GLP-1<sub>7-36amide</sub> time-averaged iAUC between conditions (Fig 2D;  $p = 0.59$ , CON ( $1.7 \pm 1.2 \text{ pmol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$ ); LOW ( $1.6 \pm 0.9 \text{ pmol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$ ); MED ( $1.4 \pm 1.1 \text{ pmol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$ ); HIGH ( $1.3 \pm 1.0 \text{ pmol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$ )). GLP-1<sub>TOTAL</sub> time-averaged iAUC did not differ between males and females ( $p = 0.90$ ), however, GLP-1<sub>7-36amide</sub> time-averaged iAUC was greater for females compared to males ( $p = 0.02$ ). There was, however, no difference between conditions for males or females, respectively, for both GLP-1<sub>TOTAL</sub> time-averaged iAUC ( $p = 0.53$ ,  $p = 0.74$ ) and GLP-1<sub>7-36amide</sub> time-averaged iAUC ( $p = 0.32$ ,  $p = 0.39$ ). Peak plasma GLP-1<sub>TOTAL</sub> concentrations were  $64 \pm 19 \text{ pmol} \cdot \text{L}^{-1}$ ,  $62 \pm 17 \text{ pmol} \cdot \text{L}^{-1}$ ,  $61 \pm 14 \text{ pmol} \cdot \text{L}^{-1}$ , and  $62 \pm 17 \text{ pmol} \cdot \text{L}^{-1}$  for CON, LOW, MED and HIGH conditions, respectively, and were not statistically different ( $p = 0.75$ ). Similarly, peak plasma GLP-1<sub>7-36amide</sub> concentrations did not differ between conditions ( $p = 0.30$ ). There was also no main effect of trial ( $p > 0.99$ ) or trial x time interaction for GLP-1<sub>7-36amide</sub>:GLP-1<sub>TOTAL</sub> ratio ( $p = 0.99$ ). Change in postprandial GLP-1 iAUC (HIGH minus CON) was not significantly correlated with either BMI (Fig 3A,  $r^2 = 0.05$ ,  $p = 0.42$ ) or Homeostatic Model Assessment of Insulin

279 Resistance (HOMA-IR; Fig 3B,  $r^2 = 0.08$ ,  $p = 0.30$ ), nor was peak GLP-1<sub>TOTAL</sub>  
 280 significantly correlated with BMI (Fig 3A,  $r^2 = 0.09$ ,  $p = 0.27$ ) or HOMA-IR (Fig 3A,  $r^2 =$   
 281  $0.02$ ,  $p = 0.63$ ).



**Figure 2.** Plasma GLP-1<sub>TOTAL</sub> (A) and GLP-1<sub>7-36amide</sub> concentrations (B) and time-averaged iAUC values for plasma GLP-1<sub>TOTAL</sub> (C) and GLP-1<sub>7-36amide</sub> (D) following ingestion of protein hydrolysate (CON), and protein hydrolysate and a low (LOW), medium (MED), and high (HIGH) dose of Capolac®. Solid and dashed lines represent individual data for males and females, respectively. Data are means  $\pm$  95% nCI,  $n = 16$ , CON = 15, LOW = 15, MED = 16, HIGH = 16. GLP-1 glucagon-like peptide-1. Significance was set at  $p \leq 0.05$ .

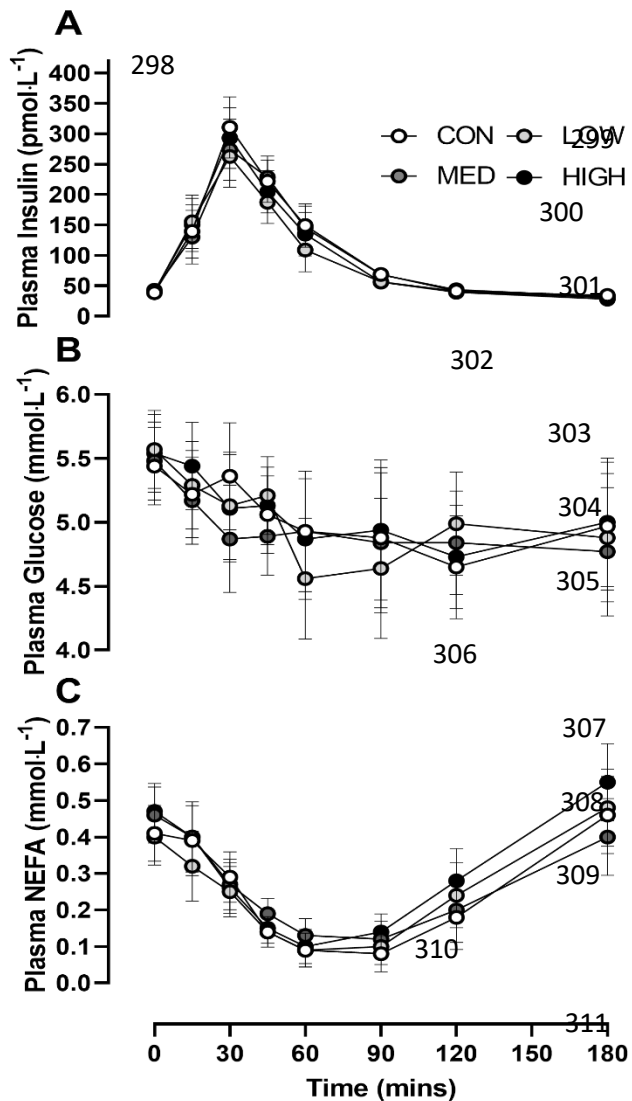


**Figure 3.** Simple linear regression for plasma GLP-1<sub>TOTAL</sub> iAUC (change from HIGH minus CON) and BMI (A) and HOMA-IR (B) and for peak plasma GLP-1 and BMI (C) and HOMA-IR (D). Filled circles and open circles represent male and females, respectively.  $n = 16$ . GLP-1 glucagon-like peptide-1. Significance was set at  $p \leq 0.05$ .

#### Plasma insulin, glucose and NEFA

There were no differences in plasma insulin ( $p = 0.51$ ), glucose ( $p = 0.94$ ), or NEFA ( $p = 0.44$ ) between conditions at baseline. Following the ingestion of test drinks, there was no effect of trial or trial x time interaction for plasma insulin (Figure 4A,  $p = 0.92$  and  $p = 0.67$ , respectively), plasma glucose (Figure 4B,  $p = 0.92$  and  $p = 0.41$ , respectively), or plasma NEFA (Figure 4C,  $p = 0.35$  and  $p = 0.11$ , respectively). The subsequent time-averaged iAUCs for plasma insulin ( $p = 0.83$ , CON ( $85 \pm 61$  pmol·L<sup>-1</sup>·min<sup>-1</sup>); LOW ( $82 \pm 96$  pmol·L<sup>-1</sup>·min<sup>-1</sup>); MED ( $86 \pm 90$  pmol·L<sup>-1</sup>·min<sup>-1</sup>); HIGH ( $83 \pm 84$

pmol·L<sup>-1</sup>·min<sup>-1</sup>)) and plasma glucose ( $p = 0.79$ , CON ( $0.10 \pm 0.25$  mmol·L<sup>-1</sup>·min<sup>-1</sup>); LOW ( $0.08 \pm 0.22$  mmol·L<sup>-1</sup>·min<sup>-1</sup>); MED ( $0.06 \pm 0.13$  mmol·L<sup>-1</sup>·min<sup>-1</sup>); HIGH ( $0.05 \pm 0.08$  mmol·L<sup>-1</sup>·min<sup>-1</sup>)), and the time-averaged tAUC for plasma NEFA ( $p = 0.57$ , CON ( $0.24 \pm 0.10$  pmol·L<sup>-1</sup>·min<sup>-1</sup>); LOW ( $0.24 \pm 0.12$  pmol·L<sup>-1</sup>·min<sup>-1</sup>); MED ( $0.24 \pm 0.00$  pmol·L<sup>-1</sup>·min<sup>-1</sup>); HIGH ( $0.27 \pm 0.08$  pmol·L<sup>-1</sup>·min<sup>-1</sup>) did not differ between conditions.

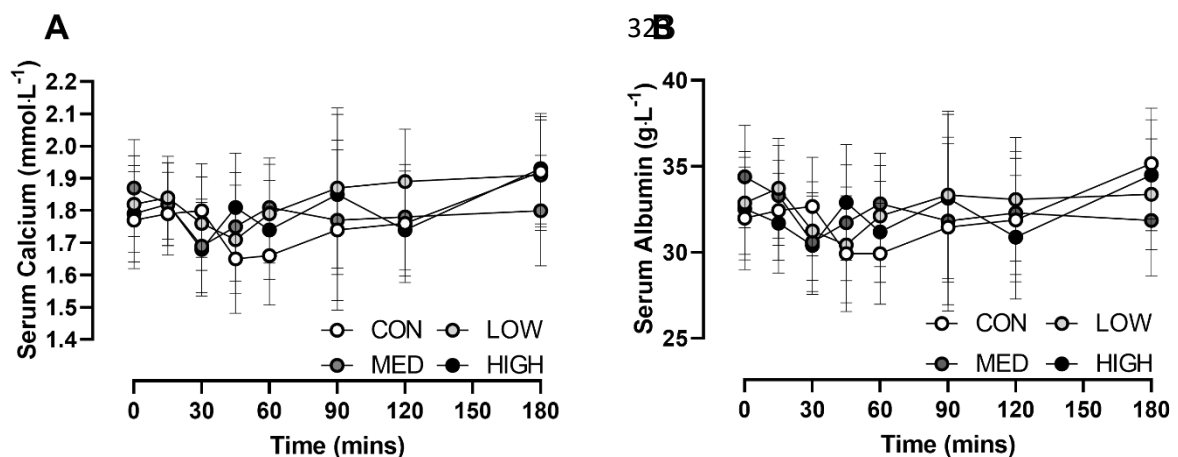


**Figure 4.** Plasma insulin (A), glucose (B) and NEFA concentrations (C) following ingestion of protein hydrolysate (CON), and protein hydrolysate and a low (LOW), medium (MED), and high (HIGH) dose of Capolac®. Data are means  $\pm$  95% nCI,  $n = 16$ . NEFA Non-esterified fatty acids. Significance was set at  $p \leq 0.05$ .



### Serum calcium and albumin

There was no effect of trial or trial x time interaction for either serum calcium (Fig 5A,  $p = 0.70$  and  $p = 0.45$ , respectively) or albumin (Fig 5B,  $p = 0.92$  and  $p = 0.41$ , respectively). There was also no differences observed in the time-averaged iAUC for serum calcium ( $p = 0.37$ , CON ( $0.10 \pm 0.14 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$ ); LOW ( $0.10 \pm 0.14 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$ ); MED ( $0.05 \pm 0.10 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$ ); HIGH ( $0.16 \pm 0.26 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$ )) or serum albumin ( $p = 0.31$ , CON ( $1.8 \pm 2.6 \text{ g} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$ ); LOW ( $1.7 \pm 2.8 \text{ g} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$ ); MED ( $0.9 \pm 2.0 \text{ g} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$ ); HIGH ( $3.1 \pm 5.2 \text{ g} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$ )) between conditions.

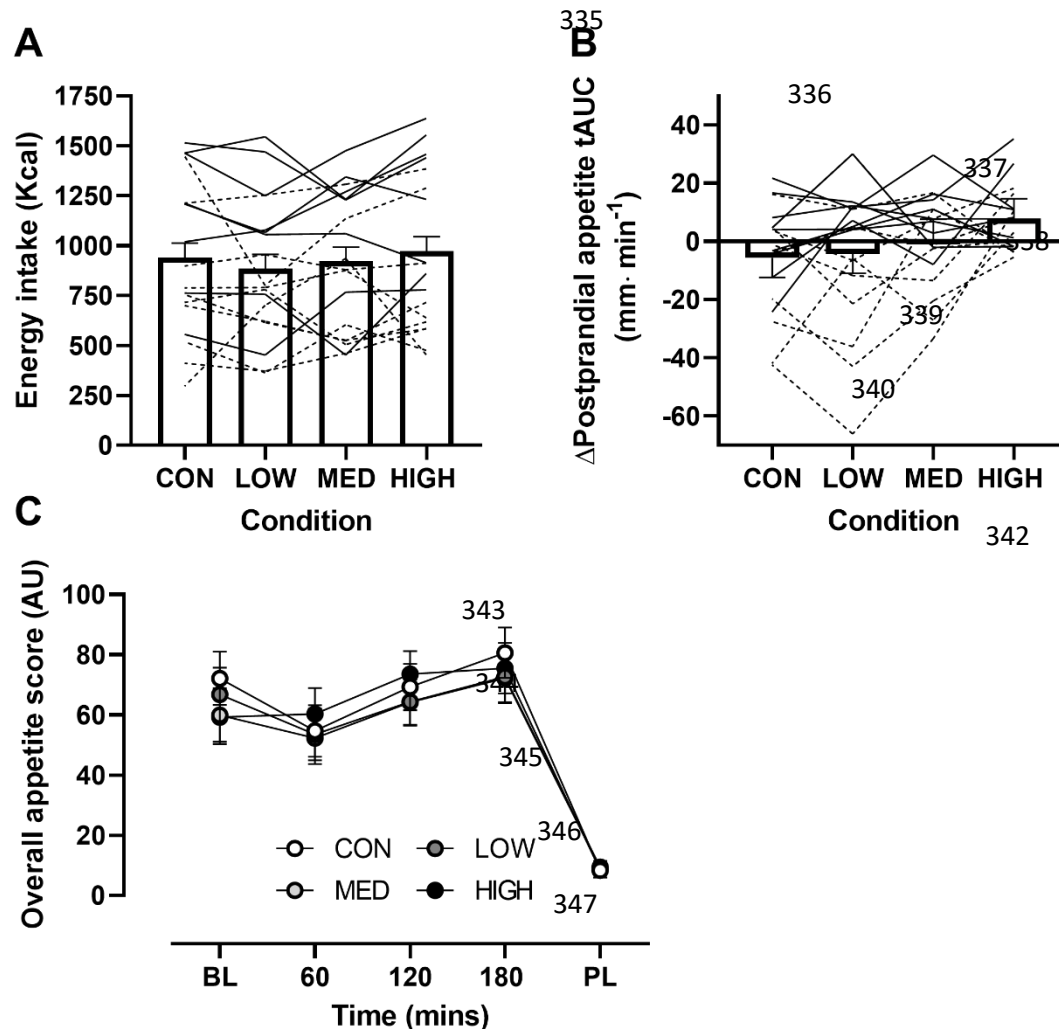


**Figure 5.** Serum Calcium (A) and albumin concentrations (B) following ingestion of protein hydrolysate (CON), and protein hydrolysate and a low (LOW), medium (MED), and high (HIGH) dose of Capolac®. Data are means  $\pm$  95% nCI,  $n = 16$ . Significance was set at  $p \leq 0.05$ .

### Ad libitum lunch and VAS

There was no difference in *ad libitum* lunch energy intake between conditions (Fig 6A;  $p = 0.35$ , CON ( $940 \pm 387 \text{ kcal}$ ); LOW ( $884 \pm 345 \text{ kcal}$ ); MED ( $920 \pm 334 \text{ kcal}$ ); HIGH ( $973 \pm 390 \text{ kcal}$ )). Equally, there were no effects for trial or trial by time interaction for

ratings of perceived fullness, satisfaction, hunger, prospective consumption or overall  
 appetite score (Fig 6C, all  $p > 0.05$ ). There was a significant difference in postprandial  
 appetite iAUC between conditions (Fig 6B,  $p = 0.04$ ), although differences did not  
 remain following *post-hoc* stepwise Holm-Bonferonni corrections.



**Figure 6.** *Ad libitum* energy intake (A), time-averaged postprandial tAUC values for appetite (B) and overall appetite score (C) following ingestion of protein hydrolysate (CON), and protein hydrolysate and a low (LOW), medium (MED), and high (HIGH) dose of Capolac®. Solid and dashed lines represent individual data for males and females, respectively. PL, post lunch. Data are means  $\pm$  95% nCI,  $n = 18$ , Significance was set at  $p \leq 0.05$ .

### Standardisation and blinding

The test drinks were well tolerated by all participants and were correctly identified on only 26% of occasions, with a third of participants unable to identify a single drink correctly. There was no difference in the time taken to ingest the test drink between conditions ( $p = 0.07$ ;  $260 \pm 80$  s,  $257 \pm 91$  s,  $264 \pm 83$  s and  $288 \pm 72$  s, for CON, LOW, MED and HIGH, respectively). Data were checked for order effects and the only parameters that exhibited any evidence of systematic variance over trial were plasma glucose and serum albumin, for which there was a main trial effect of trial sequence (both  $p = 0.03$ ), but without any sequence x trial interaction.

## DISCUSSION

The present study reveals that, contrary to our hypothesis based on previous data, the postprandial plasma GLP-1 response is not accentuated by the addition of Capolac® to ingested whey protein hydrolysate (25 g), regardless of the calcium dose across a range of 65-2547 mg. Neither plasma concentrations of insulin, glucose and NEFA nor serum concentrations of calcium and albumin differed meaningfully between conditions. There is also no effect of calcium dose on *ad libitum* energy intake from the lunch meal or overall subjective ratings of appetite.

The absence of any difference in plasma GLP-1<sub>TOTAL</sub> or GLP-1<sub>7-36amide</sub> concentrations between conditions in the present study is interesting considering these findings contradict previous work where supplementing meals with calcium enhances GLP-1 concentrations in comparison to meals without supplementation <sup>(26; 27; 28)</sup>. Furthermore, the combination of calcium and protein/amino acids has been shown to potently

stimulate GLP-1 release through co-ingestion, *in vivo* <sup>(17)</sup>, and administration, *ex vivo* <sup>(12)</sup>. The contrasting results of the present study compared to previous research may be explained by either the protein dose of the test drinks, or the population sampled in the study.

In our previous work, a large dose of whey protein hydrolysate was selected in a proof of concept study design (Chen et al., 2019). Interestingly, plasma GLP-1<sub>TOTAL</sub> time-averaged iAUC in the HIGH condition of the present study (25 g whey protein hydrolysate) was almost half the concentration ( $24 \pm 6 \text{ pmol} \cdot \text{L}^{-1} \cdot 180 \text{ min}^{-1}$ ) of that recorded following the consumption of 50 g whey protein hydrolysate (double the dose of the present study) and  $\sim 1000 \text{ mg Capolac}^{\circledR}$  ( $45 \pm 6 \text{ pmol} \cdot \text{L}^{-1} \cdot 120 \text{ min}^{-1}$ ) <sup>(17)</sup>. Equally, the protein-calcium synergy reported *ex vivo*, used isolated loops of rat small intestine where phenylalanine was perfused at a very large dose equating to  $10 \text{ mmol} \cdot \text{L}^{-1}$  <sup>(12)</sup>. Following the ingestion of a protein rich meal (50 g bovine serum albumin) in humans, ileal concentrations of phenylalanine as free and peptide amino acids reach (mean  $\pm$  SEM)  $0.36 \pm 0.08$  and  $1.65 \pm 0.33 \text{ mmol} \cdot \text{L}^{-1}$ , respectively <sup>(29)</sup>, which means enhanced stimulation of GLP-1, mediated by protein and calcium, has only been reported at high protein concentrations. Within the intestinal epithelium, L-cells capable of releasing GLP-1 increase in density along the length of the intestinal epithelium, with the greatest number located in the colon <sup>(9)</sup>. This may suggest that a larger protein dose is required to reach more distal sections of the intestine for calcium to influence GLP-1 release. Additionally, while at low doses, calcium is primarily absorbed in the duodenum and jejunum via saturable transcellular processes, at normal to high doses, the non-saturable paracellular diffusive pathway occurs throughout the intestine but dominates more distally, in the jejunum and ileum <sup>(30; 31; 32)</sup>. Considering the ingestion

of large calcium doses in the present study, the limiting factor for protein and calcium mediated GLP-1 release could be the amount of protein fed. Protein and calcium induced GLP-1 release is thought to act via the synergistic stimulation of the Calcium sensing receptor (CaSR) <sup>(12; 16)</sup>. The CaSR is able to sense amino acids in the presence of extracellular calcium concentrations of 0.5 – 10 mM <sup>(15)</sup>. Furthermore, while being directly activated by calcium, occupancy of the L-amino acid binding site enhances the sensitivity of CaSR to calcium <sup>(16; 33)</sup>, meaning that protein and calcium may work through allosteric activation of the receptor to further enhance GLP-1 concentrations. CaSR is also thought to be expressed on the basolateral membrane meaning protein absorption is paramount for GLP-1 release <sup>(13)</sup>. Therefore, insufficient protein absorption and binding may be limiting to CaSR-mediated GLP-1 release.

Another potential explanation as to why the addition of calcium did not exert the expected effect on GLP-1 could relate to the population studied. In the present study, individuals who were classified as overweight/obese were recruited to determine whether protein and calcium mediated GLP-1 release, seen in lean participants<sup>(17)</sup>, was preserved. GLP-1 release in adults with overweight and obesity has been shown to be impaired in comparison to lean controls <sup>(34; 35)</sup>, which could suggest a disruption in the signalling pathways resulting in GLP-1 release, including those involved with CaSR mediated GLP-1 release. However, these findings are inconsistent and reduced  $\beta$ -cell function, rather than a reduction in GLP-1 release, is often cited as a driver of the impaired incretin effect often observed in obese and diabetic populations <sup>(36; 37)</sup>. Moreover, in the present study there was no correlation between BMI or HOMA-IR and GLP-1<sub>TOTAL</sub> iAUC, or between BMI or HOMA-IR and peak plasma GLP-1<sub>TOTAL</sub>, suggesting that body composition and insulin sensitivity were not associated with GLP-

1 concentrations, at least in the present study. Importantly, if population differences in GLP-1 release do exist, and are mediated by factors such as BMI and insulin sensitivity, this impairment may not be linear with increasing BMI/insulin resistance but instead occur at distinct thresholds, that could not be captured in the population sampled in this study.

Despite no differences in postprandial plasma GLP-1 concentrations between conditions the average peak GLP-1<sub>TOTAL</sub> concentration for the HIGH condition ( $62 \pm 17$  pmol·L<sup>-1</sup>) was greater than those reported following the ingestion of 30 g whey protein isolate (120 kcal) in healthy older men (n = 8) and women (n = 8) ( $\sim 40$  pmol·L<sup>-1</sup>)<sup>(38)</sup> and 45 g whey protein isolate (311 kcal) in nine obese young women ( $\sim 50$  pmol·L<sup>-1</sup>)<sup>(39)</sup>. Whilst inter-study comparisons should be made with caution the higher plasma peak GLP-1 concentrations in the present study are unlikely due to the assay employed as it's been shown to have good precision and specificity for GLP-1<sub>TOTAL</sub><sup>(40)</sup>. Furthermore, fasting concentrations of GLP-1<sub>TOTAL</sub> ( $26 \pm 17$  pmol·L<sup>-1</sup>) are similar to those reported in the aforementioned studies. Greater plasma GLP-1 concentrations observed in the present study could be at least partly due to the form of whey protein ingested, whereby hydrolysed whey protein is likely to increase free amino acid exposure to the intestine<sup>(41)</sup>.

Serum calcium was not different between conditions in the present study despite such large differences in dose consumed. Calcium balance results mainly from the net effects of intestinal absorption and renal, intestinal and parathyroid gland excretion into the dominant calcium pool, bone<sup>(31; 42)</sup>. PTH and Vitamin D are two key calcium-regulating hormones, which integrate to increase bone resorption or intestinal

absorption at high and low calcium concentrations, respectively <sup>(42; 43)</sup>. These mechanisms maintain calcium concentrations within a narrow physiological range of ~2.5mM (10%) <sup>(31; 42)</sup>. The content of other minerals such as phosphorous and magnesium in each test solution differed marginally (table 2), however, this is not likely to influence gut hormone concentrations. Previously, GLP-1<sub>TOTAL</sub> iAUC was not different following ingestion of calcium citrate (< 1 mg phosphorous, < 1 mg magnesium) versus milk minerals rich in calcium (551 mg phosphorous, 26 mg magnesium) <sup>(17)</sup>.

In conclusion, the co-ingestion of a practically relevant dose of whey protein with a high dose of Capolac® did not potentiate GLP-1 release in comparison to whey protein hydrolysate alone, in people with overweight/obesity. Furthermore, *ad libitum* energy intake and subjective ratings of appetite did not differ between conditions. Future research should investigate whether there is a protein dose threshold for protein- and calcium-mediated GLP-1 release and whether this effect is specific to metabolically healthy populations or also present in clinical populations with metabolic diseases.

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